Journal of Experimental Botany, Vol. 59, No. 10, pp. 2857–2873, 2008
doi:10.1093/jxb/ern149 Advance Access publication 13 June, 2008
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Isolation of an embryogenic line from non-embryogenic *Brassica napus* cv. Westar through microspore embryogenesis

Meghna R. Malik[1], Feng Wang[1,*], Joan M. Dirpaul[1], Ning Zhou[1,†], Joe Hammerlindl[1], Wilf Keller[1], Suzanne R. Abrams[1], Alison M. R. Ferrie[1] and Joan E. Krochko[1,‡]

[1] *Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada S7N 0W9*

Received 13 February 2008; Revised 25 April 2008; Accepted 29 April 2008

Abstract

Brassica napus **cultivar Westar is non-embryogenic under all standard protocols for induction of microspore embryogenesis; however, the rare embryos produced in Westar microspore cultures, induced with added brassinosteroids, were found to develop into heritably stable embryogenic lines after chromosome doubling. One of the Westar-derived doubled haploid (DH) lines, DH-2, produced up to 30% the number of embryos as the highly embryogenic *B. napus* line, Topas DH4079. Expression analysis of marker genes for embryogenesis in Westar and the derived DH-2 line, using real-time reverse transcription-PCR, revealed that the timely expression of embryogenesis-related genes such as *LEAFY COTYLEDON1* (*LEC1*), *LEC2*, *ABSCISIC ACID INSENSITIVE3*, and *BABY BOOM1*, and an accompanying down-regulation of pollen-related transcripts, were associated with commitment to embryo development in *Brassica* microspores. Microarray comparisons of 7 d cultures of Westar and Westar DH-2, using a *B. napus* seed-focused cDNA array (10 642 unigenes), identified highly expressed genes related to protein synthesis, translation, and response to stimulus (Gene Ontology) in the embryogenic DH-2 microspore-derived cell cultures. In contrast, transcripts for pollen-expressed genes were predominant in the recalcitrant Westar microspores. Besides being embryogenic, DH-2 plants showed alterations in morphology and architecture as compared with Westar, for example epinastic leaves, non-abscised petals, pale flower colour, and longer lateral branches. Auxin, cytokinin, and abscisic acid (ABA) profiles in young leaves, mature leaves, and inflorescences of Westar and DH-2 revealed no significant differences that could account for the alterations in embryogenic potential or phenotype. Various mechanisms accounting for the increased capacity for embryogenesis in Westar-derived DH lines are considered.**

Key words: *Brassica napus*, embryogenesis, microarray, microspore, transcript profiling.

Introduction

Plant breeding is facilitated by the rapid development of doubled haploid (DH) plant lines, homozygous at all loci. This is most readily accomplished through microspore embryogenesis, where freshly isolated uninucleate or binucleate microspores (male gametophytes) are induced in culture to shift from a gametophytic or pollen pathway to embryo development. After chromosome doubling, the resultant plant lines are maintained as breeding stocks. Successful and efficient microspore-derived embryo production is genotype dependent, and much labour is consumed in developing and optimizing tissue culture conditions. Homozygous DH lines have been integrated into breeding programmes for superior varieties of canola (*Brassica* spp.), barley (*Hordeum vulgare* L.), maize (*Zea mays*), wheat (*Triticum aestivum* L.), rice (*Oryza sativa*

* Present address: National Institute for Nanotechnology, National Research Council of Canada, 11421 Saskatchewan Drive, Edmonton, Alberta, Canada T6G 2M9
† Present address: Dow AgroSciences, 9330 Zionsville Rd., Indianapolis, IN 46268, USA
‡ *Corresponding author.* E-mail: Joan.Krochko@nrc-cnrc.gc.ca

L.), pepper (*Capsicum annum*), asparagus (*Asparagus officinalis*), and several grasses such as *Lolium* and *Festuca* spp. (Thomas *et al*., 2003; Kopecky *et al*., 2005; Forster *et al*., 2007). Some selected DH lines also have been used to produce commercial hybrids, or directly as commercial lines (Forster *et al*., 2007). In addition, DH lines have become an extremely important resource for chromosome mapping studies and also facilitate selection of recessive or polygenic traits (Forster and Thomas, 2005).

Brassica napus is a model system for studies of microspore embryogenesis; however, not all genotypes respond equally well to inducing culture conditions. *Brassica napus* cv. Topas embryogenic line DH4079 is one of the most responsive genotypes, and >10% of cultured microspores form embryos (Ferrie, 2003). Some other genotypes of *B. napus*, for example cv. Allons, Garrison, and Westar, are poorly embryogenic, and <0.5% of cultured microspores form embryos using the standard *B. napus* protocol for microspore embryogenesis (Ferrie *et al*., 2005). A poor embryogenic response limits the utility of desirable cultivars in breeding programs.

Isolated microspores of suitable cultivars of *B. napus* can be induced to form embryos *in vitro* with appropriate culture media and stress treatments, for example heat (32 °C) or osmotic stress (polyethylene glycol) (Ferrie, 2003; Ferrie and Keller, 2007). Several factors are known to be critical for the optimum response of cultured microspores, including donor plant growth conditions, culture conditions, media, genotype, and age of the donor plants. Quantitative trait locus (QTL) analysis in *Brassica* cultivars for regeneration potential from protoplast cultures has revealed a low number of genes involved in this process (Holme *et al*., 2004). Zhang and Takahata (2001) have reported that microspore embryogenic ability is controlled by two multiple gene loci in *B. napus*. Also, random amplified polymorphic DNA (RAPD) markers with additive effects have been linked to microspore embryogenic ability in Chinese cabbage and oilseed rape (Zhang *et al*., 2003).

Improvements in microspore embryogenesis for poorly embryogenic cultivars of *B. napus* (i.e. cv. Westar) have been reported following additions of 24-epibrassinolide (EBR) or brassinolide to the culture medium (Ferrie *et al*., 2005). In addition, there are numerous reports in the literature of positive changes in embryogenic response in plant species and cultivars due to alterations in either donor plant conditions, media composition, or culture conditions (Li and Devaux, 2001; Croser *et al*., 2006; Kim and Moon, 2007); however, there have been no reports, or molecular studies, of cases of induced, stable, and heritable improvements in embryogenic potential in DH plant lines resulting from such manipulation of previously non-embryogenic, parental material. In the case described here, the rare embryos produced in the Westar microspore cultures following a 3 d exposure to brassinosteroid-supplemented media developed into heritably stable embryogenic lines after chromosome doubling (DH-1, DH-2, DH-3 and DH-4). Embryo development, transformation efficiency, gene expression profiles, hormone concentrations, and phenotypic differences have been compared in the non-embryogenic *B. napus* cv. Westar parental line and a Westar-derived embryogenic line, DH-2. The molecular characterization utilized a set of well-established marker genes for embryogenesis (Malik *et al*., 2007) and a newly developed *Brassica* seed-focused cDNA array (10 642 unigenes; Xiang *et al*., 2008).

Materials and methods

Plant material

Plants of *B. napus* cv. Westar were grown in 15 cm pots in a growth cabinet with a 16 h/8 h day/night photoperiod, light intensity of 400 μmol m^{-2} s^{-1}, and day/night temperatures of 20 °C/15 °C. Following flower bud formation, and in preparation for microspore culture, the day/night temperatures were lowered to 10 °C/5 °C. Microspore collections and cultures were initiated as described by Ferrie and Keller (1995). For comparison purposes, it should be noted that microspores collected from 100 buds were sufficient for ~20 culture plates. Embryogenesis was induced in microspores isolated at the late-uninucleate to early-binucleate stage (Ferrie and Keller, 1995) using a defined medium containing 13% sucrose and heat stress at 32 °C for 3 d. EBR (OlChemIm) at 10^{-6} M was added to the medium to improve the response in the poorly embryogenic cultivar Westar (Ferrie *et al*., 2005), and several of the resulting microspore-derived embryos from those cultures were regenerated into plants. The plantlets were treated with 0.34% colchicine solution for chromosome doubling in order to ensure recovery of DH plants. The colchicine-treated plantlets were transferred to soil, grown under the conditions described above, and used for subsequent microspore cultures. To assess embryogenic potential, microspores isolated from the Westar-derived DH lines (DH-1, DH-2, DH-3 and DH-4) and Westar were cultured on standard NLN-13% sucrose medium without the addition of brassinosteroids. For in-depth studies, microspore cultures of the DH-2 line and Westar were observed at 1, 3, 5, 7, 14, and 21 d for embryo development. The 5 d and 7 d microspore cultures from both lines were collected by centrifugation and stained with 1% acetocarmine to observe divisions during early embryogenesis. Light microscopy images were captured on a Leica DMR microscope.

Transformation

Agrobacterium tumefaciens strain GV3101:pMP90, carrying binary vector pHS723 which includes a β-glucuronidase–neomycin phosphotransferase (GUS–NPT) translational fusion driven by an enhanced 35S promoter with a nopaline synthase (NOS) terminator (Datla *et al*., 1991; Nair *et al*., 2000), was used for transformation. Hypocotyl explants were prepared from 5-d-old seedlings of Westar and the Westar-derived DH lines (DH-1, DH-2, DH-3 and DH-4), pre-cultured, and inoculated with *Agrobacterium* according to DeBlock *et al*. (1989), with modifications by Zou *et al*. (1997). Selection was carried out on shoot induction medium with 20 mg l^{-1} kanamycin. Green regenerants were tested for GUS activity by incubating leaf pieces in X-Gluc substrate (Jefferson *et al*., 1986).

RNA isolation

Total RNA from 0 h, 1, 3, 5, and 7 d cultured microspores was isolated using the RNeasy Midi kit (Qiagen), including on-column DNase digestion. For semi-quantitative RT-PCR, 3 μg of total RNA was used for first-strand cDNA synthesis with oligo$(dT)_{16}$ primers (DNA Sequencing Lab, NRC-Plant Biotechnology Institute) and SUPERSCRIPT II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Gene-specific primer pairs were designed using Primer 3 software (http://gene.pbi.nrc.ca/cgi-bin/primer/primer3_www.cgi) to obtain PCR products that were 350–550 bp in length. PCRs were one cycle at 95 °C for 5 min and 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s using 0.6 μl of template cDNA from the first-strand cDNA synthesis reaction.

Expression analyses by real-time RT-PCR

Total RNA (150 ng) from each tissue, developmental stage, or cultivar was used for one-step real-time reverse transcription-PCR (RT-PCR) analyses using the QuantiTect SYBR Green RT-PCR Kit (Qiagen Inc.) and gene-specific primers. Primer pairs were designed using the Primer Quest software (Integrated DNA Technologies) to give PCR products from 100 to 400 bp. Real-time RT-PCR was performed on an Mx3000P™ Real-time PCR system (Stratagene, La Jolla, CA, USA). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with 18S rRNA as the internal control gene. The cycling parameters were one cycle at 50 °C for 60 min (reverse transcription reaction), one cycle at 95 °C for 15 min, then 35 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 45 s. Primer sequences were described in a previous publication (Malik *et al*., 2007).

Microarray analysis using a B. napus *seed-focused cDNA array (Bn10K)*

For microarray analyses, the 7 d microspore cultures of Westar and the Westar-derived DH-2 line were size-selected on a mesh screen (Sefar; pore size 35 μm) in order to collect the microspores and/or cell clusters that had enlarged, and to discard the smaller physiologically unresponsive microspores. A 10 μg aliquot of total RNA from each sample was labelled with Cy3 or Cy5 (Amersham) and hybridized to the *Brassica* seed-focused cDNA microarray (Bn10K; http://brassicagenomics.ca) (Xiang *et al*., 2008) using the Pronto!™ Plus indirect system according to the manufacturer's instructions (Corning). The slides were scanned using a ScanArray 4000 laser scanner at a resolution of 10 μm. The image analysis and signal quantification were done with QUANTARRAY (GSI Lumonics, Watertown, MA, USA). Data storage and preliminary data processing, including normalization, were done on BioArray Software Environment (BASE 1.0; Saal *et al*., 2002). Background-subtracted signals were used to identify differentially expressed genes with Significance Analysis of Microarrays (SAM 2.0; Tusher *et al*., 2001).

Hormone profiling

One gram fresh weight tissue samples from young unexpanded leaves, mature leaves, and inflorescences bearing buds, with 3–4 open flowers, of matched samples of Westar and DH-2 were collected, frozen in liquid nitrogen, and freeze-dried. Three biological replicates (50 mg dry weight) were used for hormone profiling of abscisic acid (ABA) and related metabolites, auxins, and cytokinins using the protocol described in Chiwocha *et al*. (2005).

Results

Embryogenic potential of the DH lines

The DH lines (DH-1, DH-2, DH-3 and DH-4) were developed from four embryos randomly selected from Westar microspore cultures that had been previously treated with EBR to improve embryogenesis (Ferrie *et al*., 2005). In the absence of an EBR treatment, embryo development from Westar-derived microspores under standard inducing conditions was extremely rare (Table 1). The selected embryos were regenerated to plantlets, grown to flowering, and microspore embryogenesis was re-initiated in the DH lines. Surprisingly, all four of the lines had greatly improved rates of microspore embryogenesis as compared with the non-embryogenic parental line Westar (Table 1). The embryogenic response was variable among the lines, but always was consistently better than the Westar parent. The most embryogenic line, DH-2, produced thousands of embryos under standard culture conditions where Westar might only produce one embryo, and in this respect it was 30% as productive as a previously characterized highly embryogenic line, *B. napus* DH4079 (Ferrie *et al*., 2005; Malik *et al*., 2007). The DH lines were selfed (to maintain the original homozygosity) and F_2 seed was collected separately for

Table 1. *Embryogenic response and transformation potential in four independently selected Westar-derived lines*

Embryogenesis was assessed as the average frequency of embryos per plate from three replicate experiments from microspore cultures of *Brassica napus* cv. Westar and Westar-derived DH (doubled haploid) lines. On average there are ~1 000 000 microspores transferred to each plate. Transformation success was assessed by determining the number of independent GUS-positive shoots relative to the number of original *Agrobacterium*-treated explants.

B. napus, CV and line	Embryogenic response[a]	Transformation response			
		No. of explants	Green shoots	No. of GUS positives	Transformation efficiency (%)
Westar	1±0	510	72	39	7.6
Westar DH-1	40±11.9	484	49	24	3.9
Westar DH-2	2235.3±1366.5	850	8	3	0.4
Westar DH-3	131.0±99.5	510	33	17	3.3
Westar DH-4	685.3±657.4	96	26	1	1.0

[a] Mean ±SE.

each line. Plants (from F_2 seed) of the most embryogenic Westar-derived line (DH-2; Table 1) were used as donor plants for further studies of microspore embryogenesis (F_3 material). The improved embryogenic potential of the DH-2 line remained stable through at least these two subsequent generations (F_2 and F_3). Molecular and phenotypic comparisons were made between the non-embryogenic parental line, Westar, and the embryogenic Westar-derived DH-2 line.

At the time of isolation (0 h), and the beginning of culture, the microspores of both lines had a prominent nucleus and the cytoplasm was restricted to the periphery. In a previous study it was noted that enlargement of the microspore was a prominent feature of 3 d heat-stress-induced embryogenic microspores of *B. napus* Topas DH4079, and that cell and nuclear divisions were predominant in the 5 d and 7 d induced microspores, with only marginal increases in size occurring during this latter period (Malik *et al*., 2007). No differences were noted between the microspores of the Westar parental line and the Westar-derived DH-2 line during the first 3 d of culture (data not shown). Following a heat stress treatment for embryo induction (3 d at 32 °C), responding Westar and DH-2 microspores enlarged similarly, to more than double their original size, and the numbers of enlarged microspores in the two lines were equivalent (~30% of cultured microspores); however, by 5 d the induced microspores of the DH-2 line had undergone a first symmetric mitotic division (Fig. 1). In contrast, the enlarged microspores of the Westar line did not divide, although they were swollen and stained bright red with 1% acetocarmine (Fig. 1). Thereafter, the induced microspores of the DH-2 line continued to undergo random cell divisions, and by 7 d the embryogenic microspores of the DH-2 line appeared as cell clusters (globular and pre-globular embryos) with remnants of the ruptured exine still remaining on the developing embryo (Fig. 1). There were very few dividing structures in 7 d Westar cultures, and some of those dividing structures already had started to degenerate (Fig. 1).

Although *B. napus* is a model system for microspore embryogenesis, there are very few lines that are both embryogenic and transformable, for example *B. napus* DH12075 (Li *et al*., 2003) and cv. Lisandra (Fukuoka *et al*., 1998; Zhang *et al*., 2003), and thus appropriate for both genetic and reverse genetics applications. Westar is easily transformable (Cardoza and Stewart, 2004) but not highly embryogenic (Table 1; Ferrie *et al*., 2005), while *B. napus* Topas DH4079 is highly embryogenic (Malik *et al*., 2007), but not transformable using standard techniques (J Hammerlindl, unpublished data). Therefore, it was of interest to examine transformability of the Westar-derived DH lines (Table 1). Some of the lines were easily transformed with *Agrobacterium* (e.g. DH-1 and DH-3); however, the most embryogenic line (DH-2) was the least transformable of the lines developed (Table 1). This line also showed restricted organogenesis (shoot regeneration) and, therefore, perhaps transformability was limited by and could be improved with some focus on regeneration and shoot production.

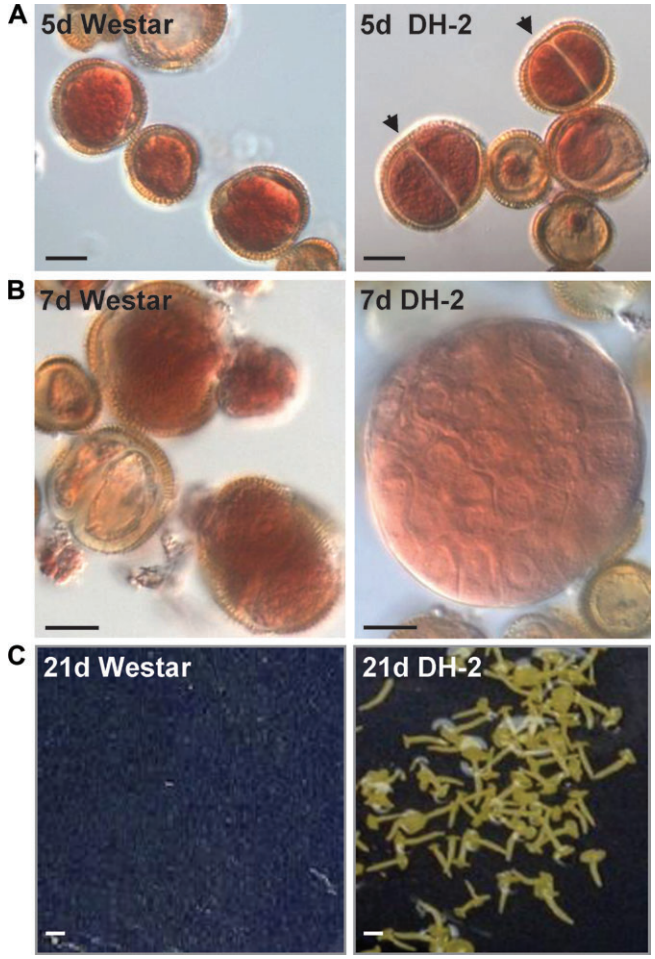

Fig. 1. Microspore-derived embryo development in *Brassica napus* cv. Westar and the Westar-derived DH-2 line. (A) Acetocarmine-stained 5 d enlarged microspores. Arrowheads indicate divisions in the microspores of the DH-2 line. (B) Acetocarmine-stained 7 d enlarged microspores in Westar and a dividing pre-globular embryo in the DH-2 line. (C) Twenty-one day mid-maturation stage embryos in the DH-2 line; no embryos developed in this microspore culture plate of Westar. Black bars=10 μm, white bars=35 μm.

Expression of embryogenesis-related genes

Previously, several clusters of differentially expressed genes marking the developmental transitions from freshly isolated microspores (0 h) to committed 7 d embryogenic cell clusters (globular and pre-globular embryos), as well as a set of 16 unambiguous marker genes for the induction of microspore embryogenesis, were identified (Malik *et al*., 2007). These molecular marker genes were not expressed in microspores at the time of culture (0 h) in the highly embryogenic *B. napus* line Topas DH4079, nor in

microspores cultured under non-inductive conditions (18 °C), and thus can be used both quantitatively and qualitatively to measure a cultivar's responsiveness to embryogenesis-inducing conditions (Malik *et al*., 2007). Based on real-time RT-PCR, the transcript abundance for the marker gene *BnLEC1* was not significantly different between the two lines at 5 d and 7 d (Fig. 2), confirming the acquisition of a certain level of embryogenic competence in cv. Westar (Fig. 1). However, *BnLEC1* expression in Westar was markedly delayed at 1 d and 3 d as compared with the DH-2 cultures (Fig. 2). Expression of the other marker genes, namely *BnLEC2*, *BnABI3*, *BnBBM1*, *BnUP1*, and *BnWOX9*, was uniformly much reduced at all time points in Westar cultures as compared with DH-2 cultures (Fig. 2), reflecting the differences in embryogenic potential between the two lines. There were no significant differences in the level of expression of *BnSERK1* between Westar and DH-2 during the early stages of microspore culture (Fig. 2), despite the marked differences in embryogenic potential.

Expression of pollen-related genes

Putative pollen-expressed genes have been identified from cDNA libraries made from 3 d and 5 d embryogenic cultures and cDNA libraries representing *in vitro* pollen (Joosen *et al*., 2007; Malik *et al*., 2007; M R Malik *et al*., unpublished data). Subsequent RT-PCR analyses have confirmed a number of predominantly pollen-expressed

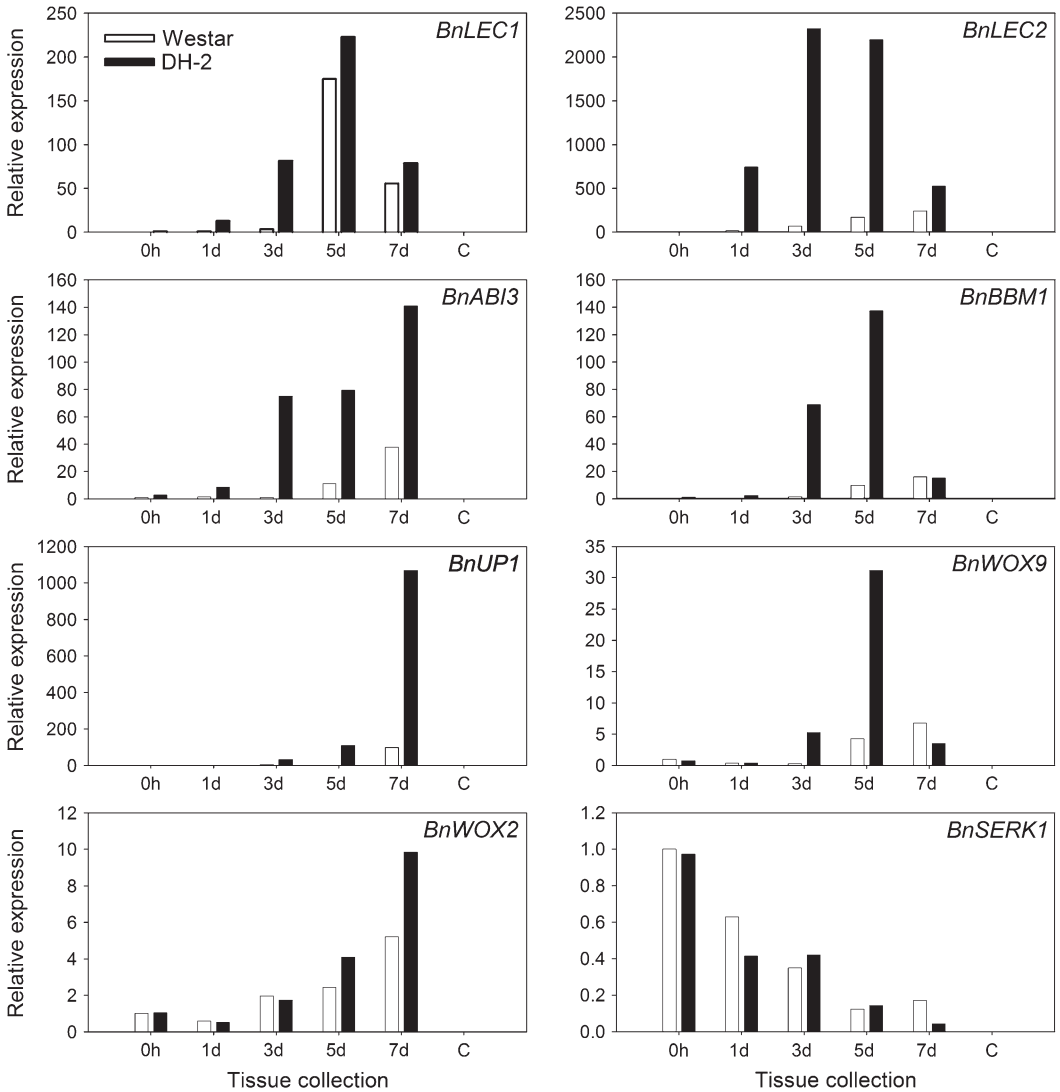

Fig. 2. Real time RT-PCR analyses of embryo-specific marker genes (*BnLEC1*, *BnLEC2*, *BnABI3*, *BnBBM1*, *BnUP1*, *BnWOX9*, and *BnWOX2*) and *BnSERK1* in microspore cultures of non-embryogenic *B. napus* cv. Westar and the embryogenic Westar-derived DH-2 line. Stages of microspore-derived embryo (MDE) development (0 h, 1, 3, 5, and 7 d) are indicated for each of the lines (Westar, DH-2). Expression was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Relative expression was based on comparisons with transcript levels in 0 h microspores of cv. Westar with 18S rRNA as the internal control for normalization.

genes, for example *BnPK12*, *BnCDPK*, *BnLEA1*, *BnPK21*, and *BnUP5* (see Supplementary Table S1 available at *JXB* online). Expression analyses by real-time RT-PCR showed at least a 2-fold greater expression of these pollen-related genes in the recalcitrant Westar cultivar, particularly at the 3 d and 5 d stages of microspore culture, as compared with the embryogenic Westar-derived DH-2 line (Fig. 3). Although the expression of these pollen-related genes diminished in cultures of both lines by 7 d, expression was still higher in the Westar microspores (Fig. 3).

Transcript profiling using the Bn10K seed cDNA microarray

Microarray analyses of gene expression using the *B. napus* 10K seed cDNA array (Xiang *et al*., 2008) identified significant expression of 637 genes in 7 d enlarged microspores of Westar and 456 genes in 7 d enlarged and dividing microspores of the DH-2 line (both samples >35 μm) and, of these, 314 genes that were expressed in both 7 d Westar and DH-2 (signal intensity >500 in two or more replicates). Using the software program SAM (Tusher *et al*., 2001), 117 differentially expressed genes were identified that mark the developmental differences between Westar and Westar-derived DH-2 (77 genes up-regulated in 7 d enlarged and dividing DH-2 microspores, 40 genes up-regulated in 7 d enlarged Westar microspores) (Fig. 4). Many of the genes up-regulated in the 7 d enlarged and dividing DH-2 microspores were related to either protein biosynthesis, response to stimulus, or cellular transport (Table 2). Notable amongst these are genes encoding 40S (RPS2C, RPS15A, RPS9B, RPSaA, RPS9B, RPS17A) and 60S (RPL10aB, RPL8A, RPL21E, RPP1B, RPL23C, RPL28C, RPL30C, RPL36aA, RPL3A) ribosomal proteins, elongation factor 2, heat shock cognate 70 kDa protein1 (HSC70-1), lipid transfer proteins, and seed storage proteins (Table 2). No differences were detected on the microarray in the levels of expression of the transcription factor genes *LEC1* and *LEC2* between the two lines, although these differences were demonstrated by real-time RT-PCR (Fig. 2), perhaps because microarray expression levels *per se* were very low for these genes (data not shown). Nonetheless, some

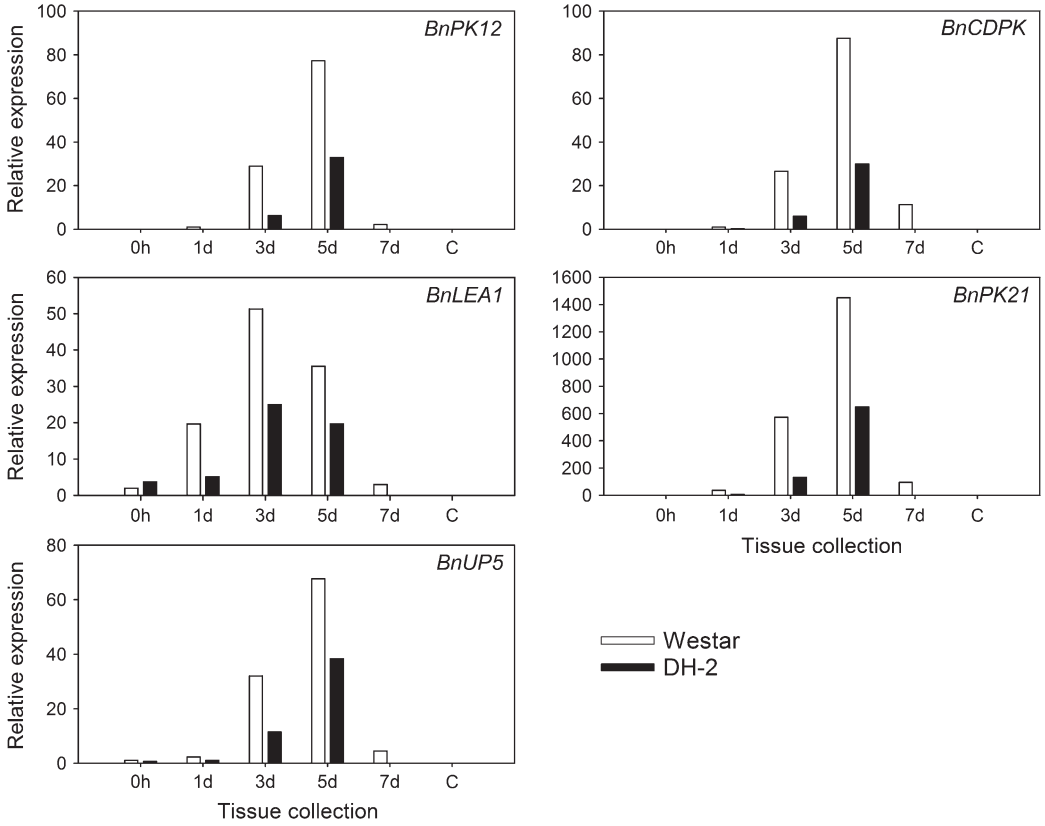

Fig. 3. Real time RT-PCR analyses of pollen-specific genes *BnPK12* (At3g18810), *BnCDPK* (At2g31500), *BnLEA1* (At4g13230), *BnPK21* (At2g24370), and *BnUP5* in microspore cultures of non-embryogenic *B. napus* cv. Westar and the embryogenic Westar-derived DH-2 line (the closest *Arabidopsis* match for each *B. napus* pollen-specific gene is given in parentheses). Stages of microspore-derived embryo (MDE) development (0 h, 1, 3, 5, and 7 d) are indicated for each of the lines (Westar, DH-2). Expression was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Relative expression was based on comparisons with transcript levels in 0 h microspores of cv. Westar with 18S rRNA as the internal control for normalization.

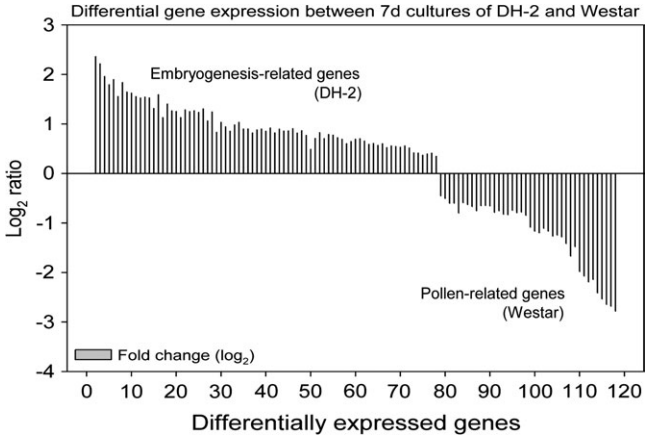

Fig. 4. Microarray analysis of differentially expressed genes between 7 d enlarged (dividing) embryogenic microspores of DH-2 and 7 d induced (non-dividing) microspores of the parental line, Westar. Labelled total RNA (10 μg) was used for hybridization to the Bn10K seed cDNA array. Signal intensities were normalized and gene lists extracted using SAM (minimum 1.5-fold change in expression). A total of 117 differentially expressed genes were identified: 77 genes up-regulated in DH-2 and 40 genes up-regulated in Westar (negative log2 values). Gene identifications are listed in Tables 2 and 3.

known targets of the LEC2 transcription factor were up-regulated and differentially expressed in the 7 d enlarged and dividing microspores of the DH-2 line, for example 2S seed storage protein 1 (At4g27140), oleosin (At4g25140), and cysteine proteinase (At3g54940) (Table 2; Braybrook *et al*., 2006).

The genes that were up-regulated in the 7 d enlarged Westar microspores (down-regulated during embryogenesis in the DH-2 line) were mostly related to pollen function, for example late embryogenesis abundant domain-containing protein/LEA (At4g13560.1), pectinesterase family protein (At3g05610), polygalacturonase (At3g07840.1), and SNF7 family protein (At5g63880.1) (Table 3). The developmental expression profile of the closest match in *Arabidopsis* for each of the 40 up-regulated genes in Westar was determined using the electronic Fluorescent Protein (eFP) Browser (http://bbc.botany.utoronto.ca/) and Genevestigator Gene Atlas (Zimmermann *et al*., 2004) on the The Arabidopsis Information Resource database (http://www.arabidopsis.org). The results of this search showed that at least 19 of these genes are highly expressed in stamen and/or pollen (Table 3).

The data indicate that induced microspores of DH-2 show up-regulation of many genes related to protein biosynthesis, while the heat-treated non-dividing microspores of Westar instead express genes related to carbohydrate and cell wall metabolism, indicative of a continued pollen developmental pathway. Additionally, some of the differentially expressed genes in induced DH-2 microspores are organellar transcripts suggestive of increased growth and multiplication of the mitochondria and chloroplasts, while no organellar transcripts were included in the list of differentially expressed genes representing Westar (pollen-like) microspores (Tables 2 and 3).

Phenotypic differences between Westar and Westar-derived DH-2 plants

In addition to the differences in embryogenic potential between Westar and DH-2, there are some striking differences in general plant morphology between these two lines (Fig. 5). The leaves of the DH-2 line are epinastic compared with leaves of Westar (Fig. 5A), and lateral branches in DH-2 are longer and more advanced than those of Westar plants of the same age, giving DH-2 plants a much bushier appearance (Fig. 5B). The petals of DH-2 plants are a paler yellow than petals of Westar flowers, and two petals of the DH-2 flowers are curled, thus imparting an asymmetry to the flower (Fig. 5C, D). The petals of DH-2 do not abscise normally, and a majority of the mature and dried siliques present retained petals, now white in colour, at the time of harvesting (Fig 5B). In addition, the uppermost portions of the inflorescences of DH-2 are looser and more elongated than the normally compact inflorescences of Westar (Fig. 5C). These features impart a different architecture to the plants and inflorescences of Westar and DH-2 lines; however, there are no differences in seed set and pollen fertility between the two lines.

Hormone profiles

It was considered that the morphological differences between the Westar and Westar-derived DH-2 plants may be related to hormone levels and, therefore, ABA, auxins, and cytokinins were profiled in young and mature leaves and inflorescences of the two lines. Determination of ABA and its metabolites showed that the highest concentrations were of dihydrophaseic acid, an oxidation product of ABA, in all three tissues examined, but especially in young leaves and inflorescences of both lines (Fig. 6). There were no notable differences in the amounts of ABA or any of its intermediates between Westar and DH-2 in the tissues examined (Fig. 6). Isopentenyladenosine (iPA) was the most abundant cytokinin detected in any of the tissues, and showed the greatest accumulation in inflorescences of Westar and DH-2 (Fig. 6). *Trans*-zeatin (t-Z), *trans*-zeatin riboside (t-ZR), *cis*-zeatin riboside (c-ZR), and isopentenyladenine (2iP) were also present in higher amounts in the inflorescences as compared with the leaves, but at much lower concentrations than iPA (Fig. 6). IAA (indole-3-acetic acid) was present in high amounts in the inflorescences, at much lower levels in young leaves, and the least amount was found in mature leaves (Fig. 6). The auxin peptide conjugates, IAA-Asp (indole-3-aspartate) and IAA-Glu (indole-3-glutamate),

Table 2. *Genes up-regulated in 7 d microspore cultures of embryogenic Westar-derived DH-2 line*

Locus identifiers for the best gene match in *Arabidopsis* are based on BlastX against the TAIR7_pep database. Biological process is taken from the Gene Ontology (GO) annotation on TAIR for each locus identifier (http://www.arabidopsis.org/tools/bulk/go/index.jsp). GenBank accession numbers identify the longest EST sequence for each gene from the collection of ESTs (~67 000 ESTs) examined to construct this cDNA array (see Xiang *et al*., 2008), and these are included in the GAL file as descriptors for each reporter (*Brassica* gene) on the microarray (http://www.brassicagenomics.ca/cdnaarray.html).
Genes listed in bold were identified previously, based on EST abundance, as up-regulated during early stages of microspore embryogenesis in *B. napus* Topas DH4079 (Malik *et al*., 2007).
Organellar-derived genes are underlined.

Accession no.	Genes up-regulated in Westar DH-2	Best match to *Arabidopsis*	E-value	Biological process	Broad functional category
EE541057	**60S ribosomal protein L32 (RPL32A)**	**AT4G18100.1**	4e-71	Ribosome biogenesis and assembly	Cell organization and biogenesis
CN735630	Histone H2B, putative	AT2G37470.1	1e-41	Chromosome organization and biogenesis	Cell organization and biogenesis
DY010080	60S ribosomal protein L5 (RPL5B)	AT5G39740.1	e-123	Ribosome biogenesis and assembly	Cell organization and biogenesis
EE542594	60S ribosomal protein L37a (RPL37aC)	AT3G60245.1	3e-49	Ribosome biogenesis and assembly	Cell organization and biogenesis
EE541600	MEE26 (maternal effect embryo arrest 26)	AT2G34870.1	4e-9	Embryonic development ending in seed dormancy	Developmental processes
DY009433	**EMBRYO DEFECTIVE 2386; identical to 60S ribosomal protein L19-1 (RPL19A)**	**AT1G02780.1**	1e-93	Embryonic development ending in seed dormancy; translation	Developmental processes; protein metabolism
CN734060	EMBRYO DEFECTIVE 2171; 60S ribosomal protein L23 (RPL23A)	AT3G04400.1	2e-76	Embryonic development ending in seed dormancy; translation	Developmental processes; protein metabolism
CN727564	EMBRYO DEFECTIVE 2171; 60S ribosomal protein L23 (RPL23A)	AT3G04400.1	4e-77	Embryonic development ending in seed dormancy	Developmental processes
EE542973	Histone H2A, putative	AT5G59870.1	8e-52	Nucleosome assembly	DNA or RNA metabolism
EE550299	NAD2B; encodes subunit of mitochondrial NAD(P)H dehydrogenase	ATMG01320.1	3e-23	Electron transport	Electron transport or energy pathways
EE550879	COB; mitochondrial apocytochrome b	ATMG00220.1	e-114	Aerobic respiration	Electron transport or energy pathways
DY007371	Mitochondrial NADH dehydrogenase 5	ATMG00665.1	7e-41	Electron transport	Electron transport or energy pathways
CN732202	**LIPID TRANSFER PROTEIN 3**	**AT5G59320.1**	1e-52	Response to abscisic acid stimulus	Other biological processes
EE548239	**Fructose-bisphosphate aldolase, putative**	**AT3G52930.1**	e-117	Pentose-phosphate shunt	Other cellular processes
CN737059	GLUTATHIONE S-TRANSFERASE 29, ATGSTU18	AT1G10360.1	4e-72	Toxin catabolic process	Other cellular processes
EE541185	MATK; encodes a maturase located in the trnK intron in the chloroplast genome	ATCG00040.1	8e-70	RNA splicing	Other cellular processes
CN727662	**GAPC-2; glyceraldehyde-3-phosphate dehydrogenase**	**AT1G13440.1**	4e-72	Gluconeogenesis	Other cellular processes
CN733306	**THI1 (THIAZOLE REQUIRING)**	**AT5G54770.1**	7e-96	Thiamine biosynthetic process	Other cellular processes
EE462458	ATP synthase beta chain 2	AT5G08690.1	1e-52	ATP biosynthetic process	Other cellular processes
CN726197	**OLEO1 (OLEOSIN1)**	**AT4G25140.1**	6e-62	Sequestering of lipid	Other metabolic processes
EE548291	**TPI; ATCTIMC (CYTOSOLIC TRIOSEPHOSPHATE ISOMERASE**	**AT3G55440.1**	1e-97	Metabolic process	Other metabolic processes
DY010101	Calmodulin binding/ elongation factor 1-alpha/ EF-1-alpha	AT5G60390.2	e-138	Translational elongation	Protein metabolism
No Acc. No.	Elongation factor 1-alpha/ EF-1-alpha	AT1G07920		Translational elongation	Protein metabolism
EE551198	RPS3; ribosomal protein S3	ATMG00090.1	e-127	Translation	Protein metabolism
EE461044	RPS3; encodes a chloroplast ribosomal protein S3	ATCG00800.1	4e-68	Translation	Protein metabolism

Continued

Table 2. *Continued*

Accession no.	Genes up-regulated in Westar DH-2	Best match to *Arabidopsis*	E-value	Biological process	Broad functional category
EE439665	Elongation factor 1-alpha/ EF-1-alpha	AT5G60390.1	7e-94	Translational elongation	Protein metabolism
EE542027	Elongation factor 1-alpha/ EF-1-alpha	AT5G60390.1	e-133	Translational elongation	Protein metabolism
EE550930	CLPP1; encodes the only ClpP (caseinolytic protease) encoded within the plastid genome	ATCG00670.1	2e-38	Proteolysis	Protein metabolism
CN725880	Cysteine proteinase, putative	AT3G54940.3	3e-85	Proteolysis	Protein metabolism
CN730121	**40S ribosomal protein S4 (RPS4B)**	**AT5G07090.1**	e-114	Translation	Protein metabolism
EE543924	RPS7, RPS7.1; encodes a chloroplast ribosomal protein S7	ATCG00900.1	1e-82	Translation	Protein metabolism
EE542577	60S ribosomal protein L36 (RPL36B)	AT3G53740.4	7e-50	Translation	Protein metabolism
EE550742	RPS6 (RIBOSOMAL PROTEIN S6)	AT4G31700.1	4e-95	Translation	Protein metabolism
CN737473	**RPS15 (RIBOSOMAL PROTEIN S15)**	**AT1G04270.1**	3e-82	Translation	Protein metabolism
CX270671	RPL2.2; encodes a chloroplast ribosomal protein L2	ATCG01310.1	1e-67	Translation	Protein metabolism
CN726155	60S ribosomal protein L10A (RPL10aB)	AT2G27530.2	e-102	Translation	Protein metabolism
EE542556	60S ribosomal protein L4/ L1 (RPL4A)	AT3G09630.1	5e-89	Translation	Protein metabolism
CN737233	60S ribosomal protein L29 (RPL29B)	AT3G06680.1	7e-31	Translation	Protein metabolism
EE551248	RPL14; encodes a chloroplast ribosomal protein L14	ATCG00780.1	4e-43	Translation	Protein metabolism
EE543194	CCB452; cytochrome c biogenesis orf452	ATMG00180.1	5e-61	Translation	Protein metabolism
DY009980	RPL2.2; encodes a chloroplast ribosomal protein L2	ATCG01310.1	e-102	Translation	Protein metabolism
CN731939	60S acidic ribosomal protein P1	AT4G00810.2	2e-29	Translational elongation	Protein metabolism
EE541984	RPS11-BETA (putative ribosomal protein S11-beta)	AT5G23740.1	6e-75	Translation	Protein metabolism
EE541830	60S ribosomal protein L10 (RPL10C)	AT1G66580.1	e-125	Translation	Protein metabolism
CN735269	**Eukaryotic translation initiation factor 5A, putative/eIF-5A, putative**	**AT1G26630.1**	3e-70	Translational initiation	Protein metabolism
EE569706	ATP1; ATPase subunit 1	ATMG01190.1	e-142	Response to oxidative stress	Response to stress
EE462567	CRT1 (CALRETICULIN 1); calcium ion binding	AT1G56340.2	e-132	Response to oxidative stress	Response to stress
DY013565	**HSP70-1, HEAT SHOCK COGNATE 70 KDA PROTEIN 1**	**AT5G02500.1**	e-116	Response to cold	Response to stress
CN730192	AT1G56075.1, LOS1 (low expression of osmotically responsive genes 1)	AT1G56070.1	7e-52	Response to cold	Response to stress
EE439511	AT1G56075.1, LOS1 (low expression of osmotically responsive genes 1)	AT1G56070.1	e-144	Response to cold	Response to stress
CN737456	**60S ribosomal protein L26 (RPL26A)**	**AT3G49910.1**	4e-58	Response to cold	Response to stress
EE541625	ATCYP1, ROC5 (ROTAMASE CYP 5)	AT4G34870.1	5e-68	Aignal transduction	Signal transduction
EE541128	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	AT5G38195.1	2e-32	Lipid transport	Transport

Continued

Table 2. *Continued*

Accession no.	Genes up-regulated in Westar DH-2	Best match to *Arabidopsis*	E-value	Biological process	Broad functional category
EE541584	**LTP12 (LIPID TRANSFER PROTEIN 12)**	**AT3G51590.1**	3e-51	Lipid transport	Transport
DY003849	2S seed storage protein 1	AT4G27140.1	1e-46	Lipid transport	Transport
DY013108	LTP2 (LIPID TRANSFER PROTEIN 2).	AT2G38530.1	2e-45	Phospholipid transfer to membrane	Transport
CN735143	**LTP5 (LIPID TRANSFER PROTEIN 5)**	**AT3G51600.1**	2e-52	Lipid transport	Transport
EE543261	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	AT1G55260.1	2e-73	Lipid transport	Transport
CN728941	AAC2 (ADP/ATP CARRIER 2)	AT5G13490.2	1e-67	Transport	Transport
EE550070	Unknown protein	AT1G49310.1	3e-23	Biological process unknown	Unknown biological processes
EE541862	Unknown protein	AT1G49310.1	9e-7	Biological process unknown	Unknown biological processes
EE543031	**BURP domain-containing protein**	**AT1G49320.1**	e-101	Biological process unknown	Unknown biological processes
EE548292	Glycine-rich protein	AT3G24250.1	9e-8	Biological process unknown	Unknown biological processes
CX271266	**BURP domain-containing protein**	**AT1G49320.1**	5e-21	Biological process unknown	Unknown biological processes
ES265407	Unknown protein	AT1G75870.1	4e-29	Biological process unknown	Unknown biological processes
EE549026	Glycine-rich protein	AT2G30560.1	2e-10	Biological process unknown	Unknown biological processes
EE542023	UInknown protein	AT3G06090.1	2e-7	Biological process unknown	Unknown biological processes
EE550161	Similar to unknown protein (*Arabidopsis thaliana*)	AT1G49290.1	3e-9	Biological process unknown	Unknown biological processes
EE548723	No hits found				
EE569070	No hits found				
EE569674	No hits found				
CN735696	No hits found				
CN730585	No hits found				
EE544345	No hits found				
CN726406	No hits found				
DY011515	No hits found				
EE569173	No hits found				

were detected at low levels in the leaves and the inflorescences (Fig. 6). In summary, no notable differences in concentrations of any of these phytohormones were found in Westar and DH-2 tissues sampled that would account for the phenotypic differences and altered embryogenic response (Fig. 6). Methods are being developed that require less tissue in order to permit routine hormone profiling of microspores, developing embryos, and microspore-derived embryos.

Discussion

The rescue of stable DH embryogenic lines from the rare embryos obtained from brassinosteroid-induced microspore cultures of the recalcitrant *B. napus* cultivar, Westar, has been described. The cultured haploid microspores bear the effects of the previous meiotic recombination, and thus collectively represent a population of all possible combinations of shuffled gene alleles prescribed within each parental genotype. Four of the rare embryos that developed from brassinosteroid-induced microspore cultures of cv. Westar were selected randomly to grow into F_1 plants, and all of these showed greatly increased rates of microspore embryogenesis in culture (Table 1) and in the later generations, as compared with the originating Westar parent. Thus, there was a direct relationship between microspores that formed embryos in the initial EBR-induced cultures, and the ability to express this characteristic heritably in succeeding generations.

The Westar-derived DH lines might have resulted from related genetic (or perhaps non-genetic) heritable changes, such as common re-arrangements or phenotypic exposure of recessive gene alleles due to fixed homozygosity at all loci. Alternatively, gene mutations should be considered. Uppermost estimates of the spontaneous mutation rate in gene-coding non-neutral DNA in plants are at 0.1–0.9 mutations per haploid genome per generation (Johnston and Schoen, 1995; Drake *et al*., 1998; Schultz *et al*., 1999). Taking the average (0.5), one would expect that

Table 3. *Genes up-regulated in 7 d microspore cultures of poorly embryogenic Westar*

Locus identifiers for the best gene match in *Arabidopsis* are based on BlastX against the TAIR7_pep database. Biological process is taken from the Gene Ontology (GO) annotation on TAIR for each locus identifier (http://www.arabidopsis.org/tools/bulk/go/index.jsp). GenBank accession numbers identify the longest EST sequence for each gene from the collection of ESTs (~67 000 ESTs) examined to construct this cDNA array (see Xiang *et al*., 2008), and these are included in the GAL file as descriptors for each reporter (*Brassica* gene) on the microarray (http://www.brassicagenomics.ca/cdnaarray.html).

Accession no.	Genes up-regulated in Westar (non-embryogenic)	Best match to *Arabidopsis*	E-value	Biological process	Broad functional category
ES264806	Pectinesterase family protein	AT3G05610.1*	2e-58	Cell wall modification	Cell organization and biogenesis
ES264447	UNE15 (unfertilized embryo sac 15)	AT4G13560.1	7e-29	Double fertilization forming a zygote and endosperm	Developmental processes
ES264881	Similar to BCP1 (*Brassica campestris* pollen protein 1)	AT3G26110.1*	1e-15	Pollen tube growth	Developmental processes
CN728581	RIC5 (ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN 5)	AT3G23380.1	4e-23	Pollen tube growth	Developmental processes
ES265290	UNE15 (unfertilized embryo sac 15)	AT4G13560.1*	5e-43	Double fertilization forming a zygote and endosperm	Other biological processes
CN728588	NHL repeat-containing protein	AT5G14890.1*	2e-84	Double fertilization forming a zygote and endosperm	Other biological processes
ES264208	Similar to unknown protein (*Arabidopsis thaliana*)	AT5G39870.1*	2e-31	Double fertilization forming a zygote and endosperm	Other biological processes
CN727705	Family II extracellular lipase	AT1G20130.1*	4e-75	Lipid metabolic process	Other metabolic processes
CN728416	ATBETAFRUCT4, VAC-INV	AT1G12240.1	e-107	Carbohydrate metabolic process	Other metabolic processes
ES264826	GDSL-motif lipase	AT5G42160.1	2e-27	Lipid metabolic process	Other metabolic processes
ES265298	Exopolygalacturonase	AT3G14040.1*	3e-43	Carbohydrate metabolic process	Other metabolic processes
ES264781	Exopolygalacturonase	AT3G14040.1*	e-101	Carbohydrate metabolic process	Other metabolic processes
ES264609	LCR1 (low-molecular-weight cysteine-rich 1)	AT5G48543.1	4e-9	Carbohydrate metabolic process	Other metabolic processes
CN728494	Polygalacturonase	AT3G07840.1*	e-110	Carbohydrate metabolic process	Other metabolic processes
CN727751	Polygalacturonase, putative	AT5G48140.1*	1e-76	Carbohydrate metabolic process	Other metabolic processes
CN727871	Protein kinase family protein	AT3G01085.1	8e-76	Protein amino acid phosphorylation	Protein metabolism
CN728491	DNAJ heat shock N-terminal domain-containing protein	AT3G04980.1	2e-53	Protein folding	Protein metabolism
CN728503	Protein kinase family protein	AT3G01085.1	2e-86	Protein amino acid phosphorylation	Protein metabolism
CN728527	BTB/POZ domain-containing protein	AT4G08455.1	3e-78	Transport	Transport
CN727745	LCR11 (low-molecular-weight cysteine-rich 11)	AT4G11485.1	4e-7	Transport	Transport
CN728424	GAMMA-TIP3/TIP1;3	AT4G01470.1*	e-117	Transport	Transport
CN727761	Amino acid permease	AT1G71680.1*	9e-87	Amino acid transport	Transport
CN728482	Encodes a maternally expressed gene (MEG) family protein	AT2G16535.1	4e-17	Amino acid transport	Transport
CN728496	SNF7 family protein	AT5G63880.1*	e-101	Protein transport	Transport
CN728126	Encodes a defensin-like (DEFL) family protein	AT4G10603.1	6e-8	Transport	Transport
CN728594	Phytochrome kinase substrate-related	AT1G18810.1	6e-12	Biological process unknown	Unknown biological processes
ES264229	Invertase/pectin methylesterase inhibitor	AT3G17220.1*	1e-63	Biological process unknown	Unknown biological processes
CN728235	Unknown protein	AT1G15415.1	7e-27	Biological process unknown	Inknown biological processes
CN728176	Similar to unknown protein (*Arabidopsis thaliana*)	AT3G28840.1*	4e-34	Biological process unknown	Inknown biological processes

Continued

Table 3. *Continued*

Accession no.	Genes up-regulated in Westar (non-embryogenic)	Best match to *Arabidopsis*	E-value	Biological process	Broad functional category
CN728423	Similar to unknown protein (*Arabidopsis thaliana*)	AT3G28790.1*	2e-31	Biological process unknown	Inknown biological processes
CN728566	Similar to unknown protein (*Arabidopsis thaliana*)	AT3G28780.1*	2e-63	Biological process unknown	Unknown biological processes
CN728544	Similar to unknown protein (*Arabidopsis thaliana*)	AT3G28790.1*	1e-16	Biological process unknown	Unknown biological processes
CN727786	Similar to unknown protein (*Arabidopsis thaliana*)	AT3G28790.1*	2e-41	Biological process unknown	Unknown biological processes
ES264118	No hits found				
ES265176	No hits found				
CN728230	No hits found				
ES264291	No hits found				
CN728497	No hits found				
ES264343	No hits found				
CN728523	No hits found				

* These genes are highly expressed in the pollen and/or stamen of *Arabidopsis* (electronic Fluorescent Protein Browser; http://bbc.botany.utoronto.ca/, Winter *et al*., 2007).

out of 1 000 000 microspores per plate, there could conceivably be a single nucleotide mutation in one gene (out of >50 000 genes) in up to half of the cultured microspores in each plate. The level could even be higher if the heat exposure induced mutations. Regardless of the mechanism, it seems reasonable to speculate that there might be a limited number of ways to overcome the block in embryogenesis genetically in cv. Westar. In addition, it seems likely that the newly acquired heritable embryogenic potential of the Westar-derived DH lines was fixed early during the first microspore culture of cv. Westar, and that it is this same capability for embryogenesis that facilitated the first rare embryogenic events in culture, and later underlies the heritable improvements in embryo production for each of the Westar-derived DH lines.

The mechanism(s) by which the applications of brassinosteroids increased rates of embryogenesis in the original microspore cultures are still unknown (Ferrie *et al*., 2005). Brassinosteroids have been shown to have several effects on tissue culture material, including stimulating cell elongation, cell division, ethylene production, adventitious tissue formation, and increased resistance to abiotic stress (Miyazawa *et al*., 2003; Mussig and Altmann, 2003; Hardtke *et al*., 2007; Kagale *et al*., 2007). Brassinosteroids have also been used to improve somatic embryogenesis in conifers (Pullman *et al*., 2003). Brassinosteroid additions were most effective in improving microspore embryogenesis in various *Brassica* species and cultivars when included in the initial media during the heat stress treatment, and were relatively ineffective when included in media after heat stress induction (A M R Ferrie *et al*., unpublished data). A lingering question is whether the brassinosteroid additions in some way caused, or affected, the heritable increase in embryogenic potential, or whether these compounds merely permitted the expression, or rescue, of those alterations.

Iterative modifications to tissue culture protocols are frequently used to improve embryogenic responses for microspores from poorly embryogenic or recalcitrant varieties, cultivars, or species; however, there have been relatively few investigations as to whether the resulting microspore-derived embryos (and perhaps newly represented genotypes) maintain the same capacity to form embryos in the next generation. In cases where microspore-derived embryos result from the acquisition of such a potential, this may be a significant way to obtain stable embryogenic lines from recalcitrant varieties. Previous reports on *Medicago* and interspecific crosses of *Helianthus* have shown that the regenerated explants from tissue culture acquired and retained the ability to respond to *in vitro* conditions and form somatic embryos in multiple subcultures (Nolan *et al*., 1989; Fambrini *et al*., 1997). In the case of Westar and the Westar-derived DH lines, 100% of the DH embryos tested (four of four) showed increased embryogenesis in the next generation as compared with Westar (Table 1).

Westar DH-2 also showed several striking differences in morphology and architecture as compared with the Westar parental cultivar. These included leaf form, axillary branching patterns, petal colour, flower symmetry, and petal abscision (Fig. 5). These abnormal phenotypes were not associated with altered concentrations of ABA, auxins, or cytokinins, or their related metabolites between Westar and DH-2 (Fig. 6). Another notable difference between the two lines was a persistent difficulty in the quantitative isolation of RNA from both microspores and early microspore-derived embryos of the DH-2 line as compared with cv. Westar and all other *Brassica* species and cultivars previously investigated (Malik *et al*., 2007). The RNA yields were up to five times less from microspores and microspore cultures of DH-2, especially from the 5 d and 7 d stages, as compared with the parent

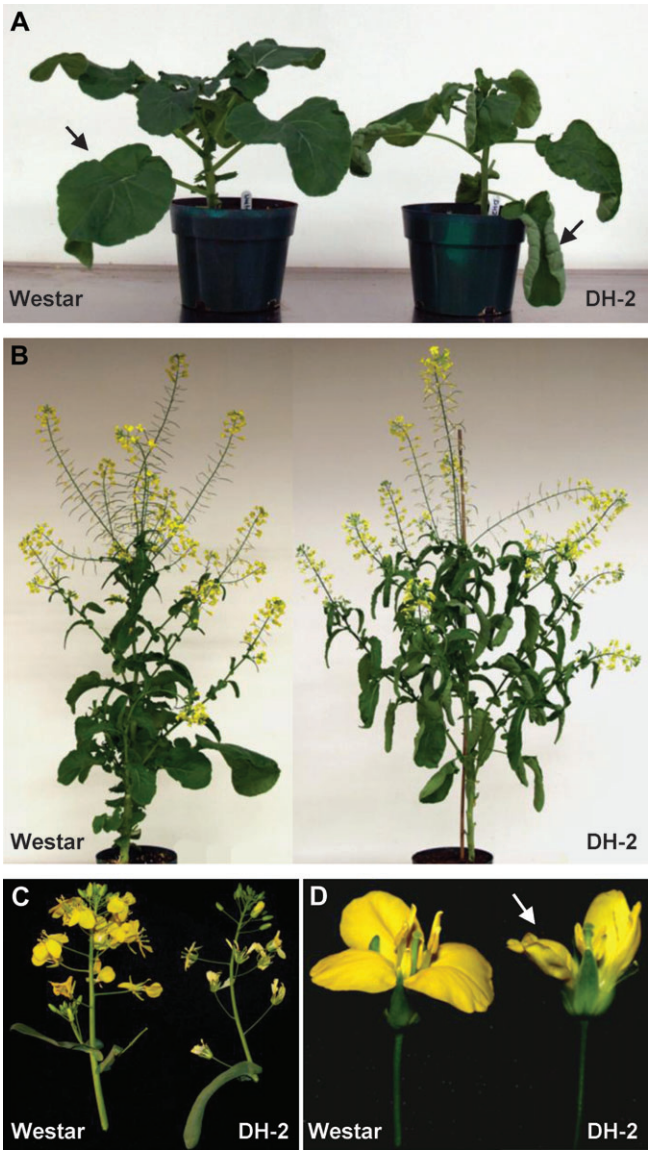

Fig. 5. Differences in morphology and architecture between plants of *B. napus* cv. Westar and the Westar-derived DH-2 line. (A) Young plants. The arrows indicate differences in leaf expansion between the two lines. (B) Plants during flowering and silique development. (C) Inflorescences. (D) Flowers on the day of anthesis. The arrow indicates wrinkled petals of the DH-2 line.

line (data not shown). There were no differences in the extractability of RNA from any other tissues of DH-2, and DNA isolation was normal in all tissues examined (data not shown).

Future studies will be necessary to determine whether any of these phenotypic characteristics between DH-2 and Westar reflect pleiotropic effects linked directly to the embryogenic potential, or instead result from additional linked genes and/or somaclonal variations induced by tissue culture. Earlier reports in *B. napus* have identified two multiple gene loci with direct effects on microspore embryogenic ability (Zhang and Takahata, 2001; Zhang *et al*., 2003). Loci with additive gene effects on *in vitro* regeneration systems have been reported for other species, *Brassica oleracea* (Holme *et al*., 2004), *Arabidopsis thaliana* (Schianteralli *et al*., 2001), *Oryza sativa* (Taguchi-Shiobara *et al*., 1997), *Solanum lycopersicum* (Koorneef *et al*., 1993), *Zea mays* (Armstrong *et al*., 1992), and *Hordeum vulgare* (Komatsuda *et al*., 1995). Genes with additive functions might account for changes in embryogenic potential as well as the accompanying phenotypic variability due to dosage effects. Similarly, chromosomal rearrangements occurring during meiosis, including translocation and homologous and homeologous recombination events, might alter gene and allelic complements in a qualitative or dosage-dependent manner, thus affecting downstream morphological and physiological outcomes. Chromosomal rearrangements during meiosis have been well documented in *B. napus* cultivars and lines, including cv. Westar (Osborn *et al*., 2003; Udall *et al*., 2005). Additionally, there are reports of other genetic- or epigenetically regulated variations caused by tissue culture (Kaeppler *et al*., 2000; Guo *et al*., 2007). Tissue culture-induced variations in barley, studied with the aid of amplified fragment length polymorphism (AFLP)-based approaches, for example, have been linked to the genotype of the donor plants, medium composition, and the length of time in culture (Bednarek *et al*., 2007, and references within). These phenotypic variations may be detrimental in micropropagation experiments, but can be exploited to good effect in other instances where they give rise to useful traits.

Microscopic observations have shown that microspores of Westar become enlarged and swollen during the first 3 d of heat stress treatment; however, only a few of the induced microspores subsequently undergo cell divisions, thus accounting for the poor embryogenic response in cv. Westar (Fig. 1). The observations suggest a block to further embryogenic development in enlarged microspores of Westar by the 5 d stage, because similar-appearing enlarged microspores of the DH-2 line show cell divisions at 5 d and continue on through embryo development (Fig. 1). Quantitative real-time RT-PCR data revealed expression of *LEC1*, *LEC2*, *ABI3*, *BBM1*, *UP1*, and *WOX9* in both Westar and DH-2, but notably that there was restricted transcription of all of these genes in the Westar cultivar, with the exception of *LEC1* (Fig. 2). Moreover, transcription of *LEC1* in 1 d and 3 d Westar cultures was delayed/inhibited, as compared with DH-2 (Fig. 2). These results provide molecular confirmation that microspores of Westar have attained some level of embryogenic competence under inductive conditions; however, this was not sufficient to drive the Westar microspores through to commitment to embryogenesis. Additionally, in conjunction with the timely and optimal expression of embryogenesis-related genes in competent microspores, pollen-expressed genes are down-regulated

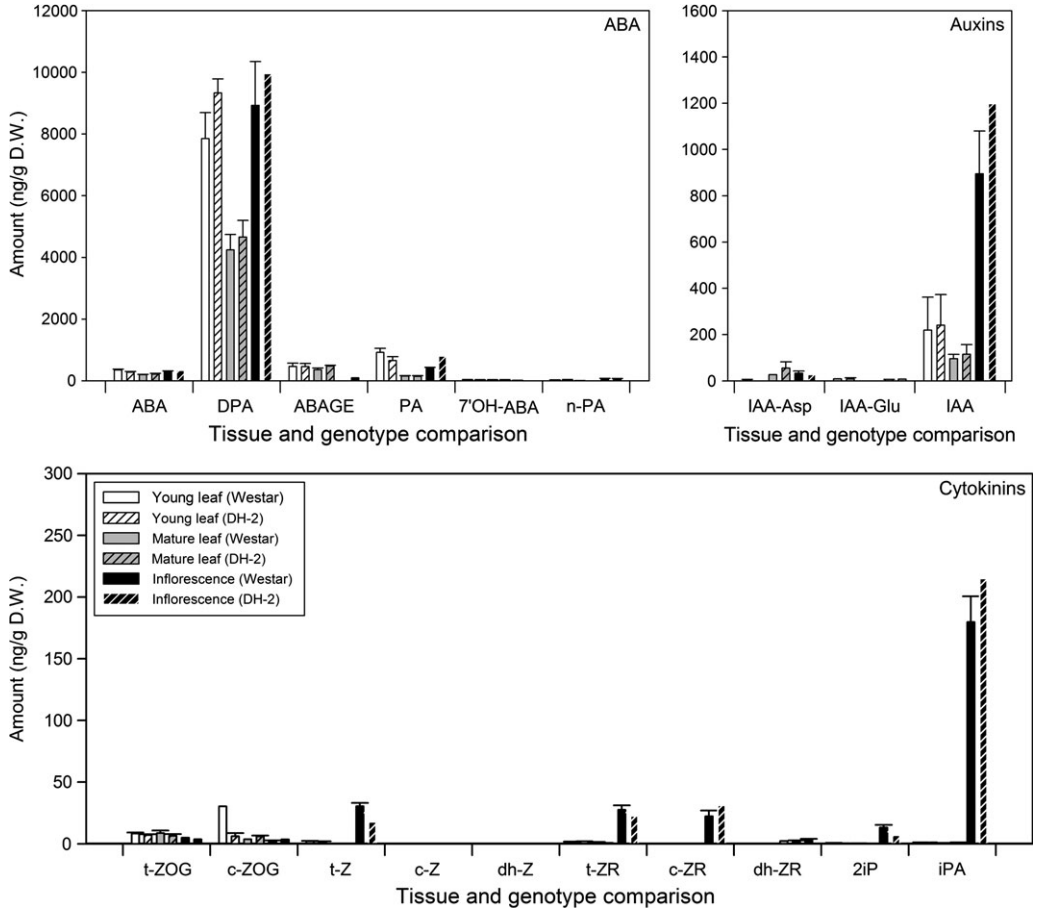

Fig. 6. Hormone profiling of metabolites and related compounds of ABA, auxin, and cytokinin in young and mature leaves and inflorescences (buds with 2–3 flowers) of *B. napus* cv. Westar and Westar-derived DH-2. Histograms indicate mean values (±SE) for each of the measured compounds, in three replicate tissue samples, each harvested from a different set of plants.

in Westar-derived DH-2, but not in cv. Westar, during the progression through to embryogenesis (Fig. 3).

So far these experiments have not revealed the nature of the molecular impediments that prevent the progression to embryogenesis in the original Westar material. To *et al.* (2006) have shown that LEC1, LEC2, ABI3, and FUS3 are major interacting and redundant regulators of embryogenesis and seed development, and each of these regulators is involved in embryo-specific transcriptional cascades affecting embryo identity and storage product accumulation (Wang *et al*., 2007). Ectopic overexpression of *LEC1*, *LEC2*, or *BBM1* is sufficient to induce embryogenesis in somatic tissues (Lotan *et al*., 1998; Stone *et al*., 2001; Boutilier *et al*., 2002), and recent studies have further demonstrated that LEC1, LEC2, and FUS3 are required for zygotic and somatic embryogenesis in *Arabidopsis* (Gaj *et al*., 2005). LEC1 and LEC2 upregulate the expression of ABI3 and FUS3 (Kagaya *et al*., 2005; To *et al*., 2006; Wang *et al*., 2007), while the regulon for LEC2 includes storage protein genes (Braybrook *et al*., 2006). The factors initially responsible for the transcriptional activation of *LEC1*, *LEC2*, and *ABI3* during heat stress-induced microspore embryogenesis in *B. napus* are not known, although there are some data to implicate alkalinization, calcium signalling, and GTPase regulation (Pauls *et al*., 2006; Chan and Pauls, 2007). More recently, a downstream involvement of auxin pools and carbohydrate metabolism in the embryogenic response, correlated with the expression of some of these transcription factors, has emerged (Casson and Lindsey, 2006). Nonetheless, there is still little information on the role of LEC1 and LEC2 (and BBM1) in potentiating an environment conducive to the induction of an embryogenic programme, or the upstream events triggering the initial transcription of these embryogenesis-related genes.

Transcript profiling using expressed sequence tag (EST) frequencies from cDNA libraries and microarray analyses have provided considerable gene expression data for various developmental stages of microspore embryogenesis in *B. napus* (Joosen *et al*., 2007; Malik *et al*., 2007; Tsuwamoto *et al*., 2007; Xiang *et al*., 2008). In the present case, microarray comparisons of Westar and Westar-derived DH-2 have provided an opportunity to examine contrasting embryogenic responses of genetically

related material cultured under identical conditions. The lists of differentially expressed genes compiled for 7 d DH-2 and Westar material clearly indicate there has been a major shift in metabolism in the DH-2 tissues during commitment to embryogenesis, involving increased translation and protein biosynthesis (Tables 2 and 3). The genes identified through the microarray comparison of Westar and DH-2 include some of the genes previously identified by EST profiling (Malik *et al*., 2007) and/or microarray analyses of highly embryogenic *B. napus* Topas DH4079 (Xiang *et al*., 2008), as well as many genes previously not listed or annotated (see Tables 2 and 3). These latter include mitochondrial- and chloroplast-derived genes, annotated embryo-defective genes, and several genes implicated in calcium responses.

In contrast, genes up-regulated in Westar include many pollen-related genes, thus underscoring the poorly embryogenic characteristics of this material (Table 3). Transcriptomic and proteomic profiling data for developing and mature pollen of *Arabidopsis* have shown a functional skew towards cell wall metabolism, carbohydrate metabolism, and cell structure (see Grennan, 2007, and references within). Surprisingly, in those same studies, genes related to protein biosynthesis were not detected in the pollen transcriptome, although their products were found in the proteome (Honys and Twell, 2004; Holmes-Davis *et al*., 2005), thereby indicating that some pollen proteins were formed early during pollen development, possibly in the uninucleate microspores which are enriched in genes related to protein biosynthesis (Malik *et al*., 2007).

It is well established that plants possess more plasticity in their genomes than most animal cells, thus allowing reprogramming of differentiated cells and expression of totipotency/pluripotency (Gutierrez, 2005; Costa and Shaw, 2007). Little is known about the details of genomic reprogramming in plant cells, but protein complexes including the Polycomb group of proteins (PcG) may be involved in the maintenance of silenced states and cellular memory. It is possible that the ability to aquire embryogenic potential or a totipotent state depends on the capacity of plant cells to modify gene expression in response to some external cues. Kinases, for example SERK (Schmidt *et al*., 1997), may be involved in the upstream perception of external stimuli, and transcription factors such as LEC1, LEC2, and BBM1 may act downstream to confer embryogenic potential (Lotan *et al*., 1998; Stone *et al*., 2001; Boutilier *et al*., 2002); however, the details of how these genes are de-repressed and the nature of their interactions to permit the expression of embryogenic potential are still not clear. Recent research progress on the stem cell potential of somatic animal tissues has taken a giant leap forward, and surged ahead of some studies in plant systems, with recent discoveries and demonstrations that expression of cassettes of four genes can transform skin cells into embryonic stem cells (Takahashi *et al*., 2007; Yu *et al*., 2007). Advances in our understanding of the cellular conditions associated with embryogenic competence and improved protocols for inducing embryogenic potential in a wide selection of plant cells will require further detailed studies of cell biology, proteomics, and metabolomics, in addition to transcript profiling. The development of genetically related lines differing in embryogenic responses, for example Westar and Westar-derived DH-2, provides an ideal opportunity for in-depth molecular studies of embryogenic potential and embryo development in plants.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Accession numbers for embryo- and pollen-specific marker genes.

Acknowledgements

This work was supported by the Genome Prairie program 'Enhancing Canola Through Genomics' through Genome Canada, a not-for-profit corporation that is leading a national strategy on genomics with $560 million in funding from the Government of Canada. We also acknowledge support from the NRC Genomics and Health Initiative II for FW and an NSERC Strategic Grant (STPGP 258143-02) to JEK. The hormone profiling studies were carried out by the Hormone Profiling Team at NRC-PBI (Sue Abrams, Irina Zaharia, Vera Cekic, Steve Ambrose; http://pbi-ibp.nrc-cnrc.gc.ca/en/research/planthormoneprofiling.htm). The authors are grateful for the critical reviews of this manuscript by Don Palmer (NRC-PBI) and Carrie-Ann Whittle (NRC-PBI). This is a National Research Council of Canada publication (NRCC #50100).

References

Armstrong CL, Romero-Severson J, Hodges TK. 1992. Improved tissue culture response of an elite maize inbred through backcross breeding, and identification of chromosomal regions important for regeneration by RFLP analysis. *Theoretical and Applied Genetics* **54,** 755–762.

Bednarek PT, Orlowska R, Koebner R, Zimny J. 2007. Quantification of the tissue-culture induced variation in barley (*Hordeum vulgare* L.). *BMC Plant Biology* **7,** 10.

Boutilier K, Offringa R, Sharma VK, *et al*. 2002. Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *The Plant Cell* **14,** 1737–1749.

Braybrook SA, Stone SL, Park S, Bui AQ, Le BH, Fischer RL, Goldberg RB, Harada JJ. 2006. Genes directly regulated by LEAFY COTYLEDON2 provide insight into the control of embryo maturation and somatic embryogenesis. *Proceedings of the National Academy of Sciences, USA* **103,** 3468–3473.

Casson SA, Lindsey K. 2006. The turnip mutant of *Arabidopsis* reveals that LEAFY COTYLEDON1 expression mediates the effects of auxin and sugars to promote embryonic cell identity. *Plant Physiology* **142,** 526–541.

Cardoza V, Stewart CN. 2004. *Brassica* biotechnology: progress in cellular and molecular biology. *In Vitro and Cellular Developmental Biology–Plant* **40,** 542–551.

Chan J, Pauls KP. 2007. *Brassica napus* Rop GTPases and their expression in microspore cultures. *Planta* **225,** 469–484.

Chiwocha SD, Cutler AJ, Abrams SR, Ambrose SJ, Yang J, Ross ARS, Kermode AR. 2005. The etr1-2 mutation in *Arabidopsis thaliana* affects the abscisic acid, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. *The Plant Journal* **42,** 35–38.

Costa S, Shaw P. 2007. 'Open minded' cells: how cells can change fate. *Trends in Cell Biology* **17,** 101–106.

Croser JS, Lulsdorf MM, Davies PA, Clarke HJ, Bayliss KL, Mallikarjuna N, Siddique KHM. 2006. Toward doubled haploid production in the Fabaceae: progress, constraints, and opportunities. *Critical Reviews in Plant Sciences* **25,** 139–157.

Datla RSS, Hammerlindl JK, Pelcher LE, Crosby WL, Selvaraj G. 1991. A bifunctional fusion between beta-glucuronidase and neomycin phosphotransferase: a broad-spectrum marker enzyme for plants. *Gene* **101,** 239–246.

DeBlock M, DeBrouwer D, Tenning P. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the bar and neo genes in the transgenic plants. *Plant Physiology* **91,** 694–701.

Drake JW, Charlesworth B, Charlesworth D, Crow JE. 1998. Rates of spontaneous mutation. *Genetics* **148,** 1667–1686.

Fambrini M, Cionini G, Pugliesi C. 1997. Acquisition of high embryogenic potential in regenerated plants of *Helianthus annuus×H. tuberosus*. *Plant Cell, Tissue and Organ Culture* **51,** 103–110.

Ferrie AMR. 2003. Microspore culture of *Brassica* species. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I, eds. *Doubled haploid production in crop plants*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 195–204.

Ferrie AMR, Dirpaul J, Krishna P, Krochko J, Keller WA. 2005. Effects of brassinosteroids on microspore embryogenesis in *Brassica* species. *In Vitro Cell and Developmental Biology–Plant* **41,** 742–745.

Ferrie AMR, Keller WA. 1995. Microspore culture for haploid plant production. In: Gamborg OL, Philips GC, eds. *Plant cell, tissue and organ culture: fundamental methods*. Berlin: Springer, 155–164.

Ferrie AMR, Keller WA. 2007. Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the Brassicaceae. *In Vitro Cellular and Developmental Biology–Plant* **43,** 348–355.

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A. 2007. The resurgence of haploids in higher plants. *Trends in Plant Science* **12,** 368–375.

Forster BP, Thomas WTB. 2005. Doubled haploids in genetics and plant breeding. *Plant Breeding Reviews* **25,** 57–88.

Fukuoka H, Ogawa T, Matsuoka M, Ohkawa Y, Yano H. 1998. Direct gene delivery into isolated microspores of rapeseed (*Brassica napus* L.) and the production of fertile transgenic plants. *Plant Cell Reports* **17,** 323–328.

Gaj MD, Zhang S, Harada JJ, Lemaux PG. 2005. Leafy cotyledon genes are essential for induction of somatic embryogenesis of *Arabidopsis*. *Planta* **222,** 977–988.

Grennan AK. 2007. An analysis of the *Arabidopsis* pollen transcriptome. *Plant Physiology* **145,** 3–4.

Guo WL, Wu R, Zhang YF, Liu XM, Wang HY, Gong L, Zhang ZH, Liu B. 2007. Tissue culture-induced locus-specific alteration in DNA methylation and its correlation with genetic variation in *Codonopsis lanceolata* Benth. et Hook. F. *Plant Cell Reports* **26,** 1297–1307.

Gutierrez C. 2005. Coupling cell proliferation and development in plants. *Nature Cell Biology* **7,** 535–541.

Hardtke CS, Dorcey E, Osmont KS, Sibout R. 2007. Phytohormone collaboration: zooming in on auxin–brassinosteroid interactions. *Trends in Cell Biology* **17,** 485–492.

Holme IB, Torp AM, Hansen LN, Andersen SB. 2004. Quantitative trait loci affecting plant regeneration from protoplasts of *Brassica oleracea*. *Theoretical and Applied Genetics* **108,** 1513–1520.

Holmes-Davis R, Tanaka CK, Vensel WH, Hurkman WJ, McCormick S. 2005. Proteome mapping of mature pollen of *Arabidopsis thaliana*. *Proteomics* **5,** 4864–4884.

Honys D, Twell D. 2004. Transcriptome analysis of the haploid male gametophyte development in *Arabidopsis*. *Genome Biology* **5,** R85.

Jefferson RA, Burgess SM, Hirsch D. 1986. β-Glucuronidase from *Escherchia coli* as a gene-fusion marker. *Proceedings of the National Academy of Sciences, USA* **83,** 8447–8451.

Johnston MO, Schoen DJ. 1995. Mutation rates and dominance levels of genes affecting total fitness in two angiosperm species. *Science* **267,** 226–229.

Joosen R, Cordewener J, Supena EDJ, *et al.* 2007. Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. *Plant Physiology* **144,** 155–172.

Kaeppler SM, Kaeppler HF, Rhee Y. 2000. Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology* **43,** 179–188.

Kagale S, Uday KD, Krochko JE, Keller WA, Krishna P. 2007. Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. *Planta* **225,** 353–364.

Kagaya Y, Toyoshima R, Okuda R, Usui H, Yamamoto A, Hattori T. 2005. LEAFY COTYLEDON1 controls the seed storage protein genes through its regulation of FUSCA3 and ABSCISIC ACID INSENSITIVE3. *Plant Cell Physiology* **46,** 399–406.

Kim YW, Moon HK. 2007. Enhancement of somatic embryogenesis and plant regeneration in Japanese larch (*Larix leptolepis*). *Plant Cell, Tissue and Organ Culture* **88,** 241–245.

Komatsuda T, Taguchi-Shiobara F, Oka S, Takaiwa F, Annaka T, Jacobsen HJ. 1995. Transfer and mapping of the shoot-differentiation locus *Shd1* in barley chromosome 2. *Genome* **38,** 1009–1014.

Koorneef M, Bade J, Hanhart C, Horsman K, Schel J, Soppe W, Verkerk R, Zabel P. 1993. Characterization and mapping of a gene controlling shoot regeneration in tomato. *The Plant Journal* **3,** 131–141.

Kopecky D, Lukaszewski AJ, Gibeault V. 2005. Reduction of ploidy level by androgenesis in intergeneric *Lolium–Festuca* hybrids for turf grass breeding. *Crop Science* **45,** 274–281.

Li H, Devaux P. 2001. Enhancement of microspore culture efficiency of recalcitrant barley genotypes. *Plant Cell Reports* **20,** 475–481.

Li H, Rimmer R, Yu M, Sharpe AG, Séguin-Swartz G, Lydiate D, Hegedus DD. 2003. Two *Brassica napus* polygalacturonase inhibitory protein genes are expressed at different levels in response to biotic and abiotic stresses. *Planta* **217,** 299–308.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25,** 402–408.

Lotan T, Ohto M, Yee KM, West MAL, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ. 1998.

Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell* **93,** 1195–1205.

Malik MR, Wang F, Dirpaul JM, Zhou N, Polowick PL, Ferrie AMR, Krochko JE. 2007. Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. *Plant Physiology* **144,** 134–154.

Miyazawa Y, Nakajima N, Abe T, Sakai A, Fujioka S, Kawano S, Kuroiwa T, Yoshida S. 2003. Activation of cell proliferation by brassinolide application in tobacco BY-2 cells: effects of brassinolide on cell multiplication, cell-cycle-related gene expression, and organellar DNA contents. *Journal of Experimental Botany* **54,** 2669–2678.

Mussig C, Altmann T. 2003. Genomic brassinosteroid effects. *Journal of Plant Growth Regulation* **22,** 313–324.

Nair RB, Joy RW, Kurylo E, Shi X, Schnaider J, Datla RSS, Keller WA, Selvaraj G. 2000. Identification of a CYP84 family of cytochrome P450-dependent mono-oxygenase genes in *Brassica napus* and perturbation of their expression for engineering sinapine reduction in the seeds. *Plant Physiology* **123,** 1623–1634.

Nolan KE, Rose RJ, Gorst JR. 1989. Regeneration of *Medicago truncatula* from tissue culture: increased somatic embryogenesis using explants from regenerated plants. *Plant Cell Reports* **8,** 278–281.

Osborn TC, Butrulle DV, Sharpe AG, Pickering KJ, Parkin IAP, Parker JS, Lydiate DJ. 2003. Detection and effects of a homeologous reciprocal transposition in *Brassica napus*. *Genetics* **165,** 1569–1577.

Pauls KP, Chan J, Woronuk G, Schulze D, Brazolot J. 2006. When microspores decide to become embryos—cellular and molecular changes. *Canadian Journal of Botany* **84,** 668–678.

Pullman GS, Zhang Y, Phan BH. 2003. Brassinolide improves embryogenic tissue initiation in conifers and rice. *Plant Cell Reports* **22,** 96–104.

Saal LH, Troein C, Vallon-Christersson J, Gruvberger S, Borg A, Peterson C. 2002. BioArray Software Environment (BASE): a platform for comprehensive management and analysis of microarray data. *Genome Biology* **3,** software0003.1–0003.6.

Schianteralli E, De la Pena A, Candela M. 2001. Use of recombinant inbred lines (RIL) to identify, locate and map major genes and quantitative trait loci involved with *in vitro* regeneration ability in *Arabidopsis thaliana*. *Theoretical and Applied Genetics* **102,** 335–341.

Schmidt ED, Guzzo F, Toonen MA, de Vries SC. 1997. A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development* **124,** 2049–2062.

Schultz ST, Lynch M, Willis JH. 1999. Spontaneous deleterious mutation in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **96,** 13393–11398.

Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada JJ. 2001. LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. *Proceedings of the National Academy of Sciences, USA* **98,** 11806–11811.

Taguchi-Shiobara F, Lin SY, Tanno K, Komatsuda T, Yano M, Sasaki T, Oka S. 1997. Mapping quantitative trait loci associated with regeneration ability of seed callus in rice, *Oryza sativa* L. *Theoretical and Applied Genetics* **95,** 828–833.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131,** 861–872.

Thomas WTB, Forster BP, Gertsson B. 2003. Doubled haploids in breeding. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I, eds. *Doubled haploid production in crop plants. A manual*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 337–349.

To A, Valon C, Savino G, Guilleminot J, Devic M, Giraudat J, Parcy F. 2006. A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. *The Plant Cell* **18,** 1642–1651.

Tsuwamoto R, Fukuoka H, Takahata Y. 2007. Identification and characterization of genes expressed in early embryogenesis from microspores of *Brassica napus*. *Planta* **225,** 641–652.

Tusher V-G, Tibshirani R, Chu G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences, USA* **98,** 5116–5121.

Udall JA, Quijada PA, Osborn TC. 2005. Detection of chromosomal rearrangements derived from homeologous recombination in four mapping populations of *Brassica napus* L. *Genetics* **169,** 967–979.

Wang H, Guo J, Lambert KN, Lin Y. 2007. Developmental control of *Arabidopsis* seed oil biosynthesis. *Planta* **226,** 773–783.

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ. 2007. An ''electronic fluorescent pictograph'' browser for exploring and analyzing large-scale biological data sets. *PLOS ONE* **2,** e718. doi:10.1371/journal.pone.0000718.

Xiang D, Datla R, Li F, *et al*. 2008. Development of a *Brassica* seed cDNA microarray. *Genome* **51,** 236–242.

Yu J, Vodyanik MA, Smuga-Otto K, *et al*. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318,** 1917–1920.

Zhang FL, Aoki S, Takahata Y. 2003. RAPD markers linked to microspore embryogenic ability in *Brassica* crops. *Euphytica* **131,** 207–213.

Zhang FL, Takahata Y. 2001. Inheritance of microspore embryogenic ability in *Brassica* crops. *Theoretical and Applied Genetics* **103,** 254–258.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. 2004. GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. *Plant Physiology* **136,** 2621–2632.

Zou J, Katavic V, Giblin ME, Barton DL, MacKenzie SL, Keller WA, Hu X, Taylor DC. 1997. Modification of seed oil content and acyl composition in the *Brassicaceae* by expression of a yeast sn-2 acyltransferase gene. *The Plant Cell* **9,** 909–923.